Plasma Membrane Ordering Agent Pluronic F-68 (PF-68) Reduces Neurotransmitter Uptake and Release and Produces Learning and Memory Deficits in Rats

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Abstract

A substantial body of evidence indicates that aged-related changes in the fluidity and lipid composition of the plasma membrane contribute to cellular dysfunction in humans and other mammalian species. In the CNS, reductions in neuronal plasma membrane order (PMO) (i.e., increased plasma membrane fluidity) have been attributed to age as well as the presence of the β-amyloid peptide-25-35, known to play an important role in the neuropathology of Alzheimer's disease (AD). These PMO increases may influence neurotransmitter synthesis, receptor binding, and second messenger systems as well as signal transduction pathways. The effects of neuronal PMO on learning and memory processes have not been adequately investigated, however. Based on the hypothesis that an increase in PMO may alter a number of aspects of synaptic transmission, we investigated several neurochemical and behavioral effects of the membrane ordering agent, PF-68. In cell culture, PF-68 (nmoles/mg SDS extractable protein) reduced [^{3}H]norepinephrine (NE) uptake into differentiated PC-12 cells as well as reduced nicotine stimulated [^{3}H]NE release. The compound (800–2400 μg/kg, i.p., resulting in nmoles/mg SDS extractable protein in the brain) decreased step-through latencies and increased the frequencies of crossing into the unsafe side of the chamber in inhibitory avoidance training. In the Morris water maze, PF-68 increased the latencies and swim distances required to locate a hidden platform and reduced the time spent and distance swam in the previous target quadrant during transfer (probe) trials. PF-68 did not impair performance of a well-learned working memory task, the rat delayed stimulus discrimination task (DSDT), however. Studies with ^{14}C-labeled PF-68 indicated that significant (pmoles/mg wet tissue) levels of the compound entered

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the brain from peripheral (i.p.) injection. No PF-68 related changes were observed in swim speeds or in visual acuity tests in water maze experiments, rotorod performance, or in tests of general locomotor activity. Furthermore, latencies to select a lever in the DSDT were not affected. These results suggest that PF-68 induced deficits in learning and memory without confounding peripheral motor, sensory, or motivational effects at the tested doses. Furthermore, none of the doses induced a conditioned taste aversion to a novel 0.1% saccharin solution indicating a lack of nausea or gastrointestinal malaise induced by the compound. The data indicate that increases in neuronal plasma membrane order may have significant effects on neurotransmitter function as well as learning and memory processes. Furthermore, compounds such as PF-68 may also offer novel tools for studying the role of neuronal PMO in mnemonic processes and changes in PMO resulting from age-related disorders such as AD.

Introduction

The most commonly perceived function of the plasma membrane in living cells is that of a physical barrier between the intracellular environment and the external milieu. Historically, the plasma membrane has been considered an inert "lipid scaffold structure" in which the elements essential for transmembrane signaling (i.e., proteins) are housed and supported while they carry out their signal tranduction function (Brown and London 1998). However, it seems unlikely that the large heterogeneity observed in the lipid components of the plasma membrane would be required if it simply served only a physical barrier function. It is the possibility that transmembrane signaling can be altered by direct modification of the physical properties of the lipid matrix, rather than by altering the function of the proteins embedded in that matrix, that concerns this report.

In the past we and others have shown that the physical properties of the plasma membrane play an important role in the ability of a cell to withstand external mechanical forces (Ramirez and Mutharasan 1990, 1992; Clarke and McNeil 1992; Tomeczkowski et al. 1993; Clarke et al. 1995). The physical properties of the plasma membrane are dictated by the structure and composition of its three-dimensional lipid matrix and can be described by terms such as rigidity, elasticity, fluidity, and tensile strength, all of which are reflected in a composite descriptor of membrane physical properties known as plasma membrane order (PMO) (Needham and Evans 1988; Needham and Nunn 1990). The neutral lipid, cholesterol, is the major structural lipid present in the mammalian plasma membrane and is responsible for the mechanical stability and cohesiveness of the membrane because of its membrane ordering ability (Yeagle 1985). In addition, it is the presence of cholesterol in mammalian plasma membranes that confers its "liquid crystal" or "liquid-ordered phase" at physiological temperatures (i.e., 37°C) (Brown and London 1998). For example, a small increase in membrane cholesterol content results in an increase in membrane rigidity and decrease in membrane fluidity (Evans and Rawicz 1990), whereas it also decreases the susceptibility of mammalian cells to mechanically induced plasma membrane damage (Ramirez and Mutharasan 1992; Tomeczkowski et al. 1993). Conversely, a large increase in plasma membrane cholesterol content results in large increases in both PMO and membrane rigidity, decreased plasma membrane fluidity, and a precipitous increase in susceptibility to mechanically induced plasma membrane damage (Tomeczkowski et al. 1993; Clarke et al. 1995). Similar results using synthetic plasma membrane ordering and disordering agents have confirmed the relationship between PMO, plasma membrane fluidity, and altered susceptibility to mechanically induced plasma membrane damage (Clarke and McNeil 1992; Clarke et al. 1995).

Experimental data from a variety of scientific disciplines and proposed mathematical models indicate that an optimal level of membrane order is essential for normal cell function and that either positive or negative changes in membrane order can have deleterious effects (DeLisi and Wiegel 1983; Tanii et al. 1994; Ryan et al. 1996; Gimenez 1998; Whiting et al. 1998). For example, increased PMO (i.e., decreased plasma membrane fluidity) and increased membrane cholesterol content have been linked to a variety of disease states including atherosclerosis and diabetes mellitus (Watala et al. 1987; Gillies and Robinson 1988; Winocour et al. 1990). In addition, other evidence strongly suggests that alterations in PMO brought about by changes in plasma membrane cholesterol content directly impact transmembrane signaling by alter-

ing membrane protein function. These effects have been attributed to bulk lipid loading of the membrane (i.e., increased PMO due to increased packing of the interphospholipid spaces with cholesterol), by direct interaction of cholesterol with the membrane protein resulting in disruption of the ability of the protein to undergo the conformational changes required for signaling (i.e., "molecular freezing"), or by a combination of both phenomena (for review, see Bastiaanse et al. 1997). Paradoxically, the effect of cholesterol enrichment on plasma membrane protein function can be either stimulatory, as in the case of cholesterol-enriched, vascular smooth or cardiac muscle cell DHP-sensitive calcium channel activity (Bialecki and Tulenko 1989; Gleason et al. 1991; Bastiaanse et al. 1994), or inhibitory, as in the case of sarcolemma (Ca^{2+}, Mg^{2+})-ATPase activity (Ortega and Mas-Oliva 1984) and Ca^{2+}-dependent K^+ channels (Bolotina et al. 1989).

A number of reports have documented decreases in platelet PMO (i.e., increases in plasma membrane fluidity) in Alzheimer's disease (AD) patients (for review, see Zubenko et al. 1996). This phenomenon has also been observed in leukocytes in AD patients and in older patients with Down's syndrome (Scott et al. 1994). The β-amyloid peptide (25-35), known to play a significant role in the pathogenesis of AD, has been shown to increase PMO (i.e., decrease membrane fluidity) in the cortex, hippocampus, striatum, and cerebellum in rats (Muller et al. 1995) as well as the frontal and parietal cortex of humans (Muller et al. 1998). The authors suggested that these effects of β-amyloid (A beta) on neuronal membranes probably play a major role in the initiation of events leading to the neurotoxicity and cell death of AD. Furthermore, Stoll and colleagues (1996) found that gingko biloba, a plant extract associated with increased cognitive function in humans, appears to have a direct neuronal membrane disordering effect.

Recent studies have also indicated that nicotinic acetylcholine receptors (nAChRs) require optimum PMO as well as the presence of both neutral and anionic lipids to retain normal flux and desensitization capabilities. These receptors are well known to play an important role in learning and memory and general cognitive function (Terry et al. 1997; for review, in Levin and Simon 1998). It appears that in the absence of optimal membrane lipid content (and thus optimal membrane order), the nAChR adopts a channel inactive conformation analogous to the ligand-induced desensitized state (for review, see Ryan et al. 1996). This phenomenon could have very important ramifications for age-related disorders or memory. The role of neuronal PMO in synaptic transmission in the CNS especially as it relates to learning and memory has not been extensively investigated, however. Based on the hypothesis that an increase in PMO (i.e., a decrease in plasma membrane fluidity) alters several processes important to synaptic transmission, we investigated the neuronal and mnemonic effects of a synthetic membrane ordering agent, Pluronic F-68 (a.k.a., Polaxmer 188). This compound (PF-68) is a nonionic surfactant molecule shown previously to intercalate into the plasma membrane of mammalian cells in culture and directly increase PMO (Ramirez and Mutharasan 1990; Clarke et al. 1995). We thus studied the effects of PF-68 on neuronal function both in vitro and in vivo. Using a tissue culture model (the rat PC-12 cell line) (Terry and Clarke 1994) and several behavioral tasks in the whole animal, we tested the hypothesis that increasing neuronal PMO using PF-68 would lead to neuronal membrane dysfunction and a perturbation of neuronal transmission at the cellular level (in vitro) and that disruption of such cellular effects in vivo would underlie decrements in cognitive ability in rats as demonstrated by impaired performance of memory related tasks.

Materials and Methods

BRAIN PENETRATION STUDY

To determine the concentration of PF-68 that penetrated the brain from peripheral injection, four rats were administered a dose of 2400 μg/kg of ^{14}C-labeled PF-68 (0.24 mCi/gram; Cytrex Corporation, Atlanta, GA) intraperitoneally (i.p.) dissolved in phosphate buffered saline (PBS). The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) 30 min later, and the brains were subsequently perfused transcardially with 100 ml of PBS as described previously (Yells et al. 1994). The brains were subsequently removed, the hippocampus, frontal cortex, corpus striatum and cerebellum were dissected from two of the brains and homogenized (in PBS), and the other two brains were homogenized whole. An aliquot from each homogenate containing 50 mg of wet tissue was subsequently added to 5.0 ml of scintillation fluid. After 4 hr, ^{14}C radioactivity was quantitated in a scintillation counter (Beckman model LS 6000TA, Beckman Instrument, Inc., Schaumburg, IL) with

counting efficiency ~45%. The moles of ^{14}C-labeled PF-68 per mg of wet tissue were back-calculated from the DPM measured. To make a meaningful comparison between in vivo and in vitro PF-68 doses, a 50-mg sample of wet brain tissue (frontal cortex) was repeatedly homogenized in 1 ml of 0.1% (wt/vol) SDS over a period of 1 hr, centrifuged at 10,000*g* for 20 min at 4°C, and the supernatant was then assayed for total protein content using the BCA total protein assay kit as per the manufacturer's instructions (Sigma Chemical Company, St Louis, MO). The amount of PF-68 present in different regions of the brain was then expressed as moles of ^{14}C-labeled PF-68 per mg of SDS extractable protein.

TISSUE CULTURE EXPERIMENTS

PC-12 cells were maintained in tissue culture as described previously (Terry and Clarke 1994). Cells were seeded at a cell density of 30,000 cells/cm^2 on poly-L-lysine-coated tissue culture plates and cultured for 7 days in standard growth medium (SGM) containing 100 ng/ml NGF to induce neuronal differentiation.

CALCULATION OF PF-68 DOSE IN DIFFERENTIATED PC-12 CELL CULTURES

Differentiated PC-12 cells were incubated for 5 min in sfSGM containing 1% (wt/vol) solution of ^{14}C-labeled PF-68 for a period of 5 min followed by washing for 5 min in sfSGM. The cells were then scraped off the culture substratum and collected by centrifugation. The cell pellet was repeatedly homogenized in 1 ml of 0.1% (wt/vol) SDS over a period of 1 hr and centrifuged at 10,000*g* for 20 min at 4°C. A 20-µl aliquot of the supernatant was assayed for total protein content using the BCA total protein assay kit as per the manufacturer's instructions (Sigma Chemical Company, St Louis, MO), whereas the remainder of the supernatant was added to 5.0 ml of scintillation fluid. After 4 hr, ^{14}C radioactivity was quantitated in a scintillation counter (Beckman model LS 6000TA, Beckman Instrument, Inc., Schaumburg, IL) with counting efficiency ~45%. The moles of ^{14}C-labeled PF-68 per mg of SDS extractable protein in the cell pellet were back-calculated from the DPM measured.

[^{3}H]NOREPINEPHRINE RELEASE ASSAY

Stimulated release of [^{3}H]norepinephrine (NE) from differentiated PC-12 cells was performed as described previously (Ikemoto et al. 1997) with the following modifications: Serum free SGM containing 25 mM HEPES and 1 mM sodium ascorbate (sfSGM) was substituted for Krebs-Ringers solution. Cells were incubated at 37°C in 5 µCi [^{3}H]NE/ml for a total of 22 min, the first 2 min of which were in the presence of 50 mM KCl to induce depolarization-sensitive vesicle fusion with the plasma membrane, rather than a 2-hr incubation with 0.4 µCi [^{3}H]NE/ml. Cells were then washed with four changes of warm sfSGM over a period of 30 min to remove free [^{3}H]NE. Alternatively, cells were washed with two changes of warm sfSGM for 15 min, incubated at 37°C with sfSGM containing 1% PF-68 (wt/vol) for 10 min followed by washing with warm sfSGM for an additional 5 min to remove free PF-68. Cells were then incubated at 37°C for 2 min in 1 ml of sfSGM after which time an aliquot (i.e., 750 µl) of medium was removed and centrifuged at 10,000*g* to remove any cellular material. The remainder of the medium was removed and replaced with 1 ml of sfSGM (pH 7.2) containing 200 µM nicotine base. The cells were incubated at 37°C for a further 2 min in this medium after which time an aliquot (i.e., 750 µl) of medium was removed and centrifuged at 10,000*g* to remove any cellular material. The amount of radioactivity in equal amounts of medium harvested before and after nicotine stimulation was determined using liquid scintillation counting. The amount of [^{3}H]NE released as a consequence of nicotine stimulation was expressed as the (%) difference from basal [^{3}H]NE release in each individual culture thereby eliminating any error introduced by differences in cell number between cultures.

[^{3}H]NE UPTAKE ASSAY

Uptake of [^{3}H]NE into differentiated PC-12 cells was carried out as described previously (Ikemoto et al. 1997). Briefly, PC-12 cells were incubated for 10 min at 37°C in sfSGM alone or sfSGM containing 1% PF-68 (wt/vol). The cells were then washed three times with warm sfSGM over a period of 5 min and then incubated with sfSGM containing 5 µCi [^{3}H]NE/ml for 5 min at 37°C. The cells were then rapidly washed four times with warm sfSGM, aspirated to dryness, and solubilized using 2% Triton X-100 (vol/vol) (aq). Total protein content per well was determined using the BCA assay, and the radioactivity in the remaining cell lysates was determined by liquid scintillation

counting. [^{3}H]NE uptake was expressed as fentamoles of [^{3}H]NE per ng of total protein.

FM I-43 RELEASE ASSAY

FM I-43 dye release was determined using a modified version of the protocol described previously (Reuter 1995). Briefly, PC-12 cells were incubated with warm sfSGM containing 50 mM KCl and 10 μM FM I-43 dye for 1 min. This was replaced with warm sfSGM containing 10 μM FM I-43, and the cells were incubated for a further 2 min. The cells were then washed three times over 20 min with warm sfSGM and then incubated with 1 ml of sfSGM (pH 7.2) containing 200 μM nicotine base. The medium was collected after 2 min, centrifuged at 10,000*g* to remove any cellular debris, and the amount of fluorescence present in a 200-μl aliquot of medium was determined using a fluorescent plate reader. Cells were then allowed to recover in fresh SGM for a period of 4 hr, at which time the cells were treated as above except that prior to stimulation with nicotine, but after FM I-43 loading, the cells were incubated in 1% (wt/vol) PF-68 for 10 min followed by washing with warm sfSGM to remove free PF-68. The amount of nicotine-stimulated FM I-43 dye released from the same cultures was determined and expressed as a fluorescence value at an excitation wave length of 530 nm and an emission wave length of 620 nm.

BEHAVIORAL EXPERIMENTS

ANIMALS

Male Wistar rats, 45 days old, were obtained from Harlan Sprague-Dawley, Inc. and housed individually in stainless steel mesh cages in a temperature controlled room (25°C) with a 12-hr light/dark cycle. Upon arrival, each animal was provided with water and standard rodent chow (NIH-07 formula) ad libitum. For delayed stimulation discrimination testing and conditioned taste aversion experiments, rats were subsequently food or water restricted (see below).

PASSIVE (INHIBITORY) AVOIDANCE TESTING

Training was conducted in a dimly lit room with a shuttle cage (Coulbourn Instruments, Lehigh Valley, PA) having a retractable (guillotine) door dividing the cage into two equal compartments. Rats were placed in this room each day, 30 min before the beginning of the experiment. Saline or PF-68 (dissolved in PBS) was administered i.p. to the rats in doses of 200, 800, or 2400 μg/kg, 30 min before testing. A training trial was initiated by placing a rat into the left (safe) compartment and fastening the top of the apparatus to prevent escape. Approximately 20 sec later a bright light came on in this side of the chamber, and the guillotine door was raised allowing for entry into the dark (unsafe) compartment. When the rat completely crossed into the dark compartment, the guillotine door immediately lowered and an inescapable, 0.8-mA (scrambled) footshock was delivered through the grid floor for 5 sec. The rat was then removed from the apparatus and returned to its home cage. Forty-eight hours later a retention trial was performed in a similar manner as the training trial except that no injections were given and no shock was delivered if the rat crossed into the unsafe compartment. The latency to enter the dark chamber (step-through latency) and learning frequencies (number of rats not crossing into the unsafe side) during the retention trial were used as measures of memory for the training experience. Rats that did not cross over into the dark chamber after 300 sec were given a latency score of 300 sec and designated as having learned the task (for frequency analysis).

SPATIAL LEARNING: MORRIS WATER MAZE

TESTING APPARATUS

Water maze testing was performed in a circular pool (diameter, 180 cm; height, 76 cm) made of plastic (Bonar Plastics, Noonan, GA) with the inner surface painted black. The pool was filled to a depth of 35 cm of water (maintained at 25.0 ± 1.0°C) that covered an invisible (black) 10-cm square platform. The platform was submerged ~1.0 cm below the surface of the water and placed in the center of the northeast quadrant. The pool was located in a large room with a number of extramaze visual cues including highly reflective geometric images (squares, triangles, circles, etc.) hung on the wall, diffuse lighting, and black curtains used to hide the experimenter and the awaiting rats. Swimming activity of each rat was monitored via a ccTV camera mounted overhead, which relayed information including latency to find the platform, total distance traveled, time and distance spent in each quadrant, etc., to a video tracking

system (Poly-Track, San Diego Instruments, San Diego, CA). Tracking was accomplished via a white rat on a black background.

PROCEDURE

Saline or PF-68 (in PBS) was administered i.p. to the rats in doses of 200, 800, or 2400 μg/kg 30 min before testing in the maze. Each rat was given four trials per day for 4 consecutive days to find the hidden platform. A trial was initiated by placing the rat in the water facing the pool wall in one of the four quadrants (designated NE, NW, SE, SW). The daily order of entry into individual quadrants was randomized such that all four quadrants were used once every day. For each trial, the rat was allowed to swim a maximum of 90 sec, to find the platform. When successful, the rat was allowed a 30-sec rest period on the platform. If unsuccessful, within the allotted time period, the rat was given a score of 90 sec and then physically placed on the platform and also allowed the 30-sec rest period. In either case the rat was immediately given the next trial after the rest period. On day 5, two probe trials (transfer tests) were given in which the platform was removed from the pool to measure "spatial bias" (Morris 1984). This was accomplished by measuring the time and distance traveled in each of the four quadrants. Immediately following the transfer test, the platform was reintroduced to the pool in the quadrant diametrically opposite the original position (SW quadrant) with a highly visible (light reflective) cover attached to the platform that was raised above the surface of the water (~1.5 cm). Lighting was changed such that extramaze cues were no longer visible and a spotlight illuminated the visible platform only. Each rat was given one trial to acclimate to the new set of conditions and locate the platform visually. This was accomplished by lowering the rat into the water in the NE quadrant and allowing location of the platform. No time limit was placed on this first trial. The rat was then immediately given a second trial in the same manner, and the latency to find the platform was measured as a comparison of visual acuity.

WORKING MEMORY: RAT DELAYED STIMULUS DISCRIMINATION TASK

Upon arrival, each rat used for delayed stimulus discrimination task (DSDT) testing was provided with water and standard rodent chow (NIH-07 formula) ad libitum for 1 week. They were restricted during the next week to a daily feeding of 18 grams per day (~80% of their ad libitum consumption). Additional food was given on weekends and holidays to maintain the weight of each rat at approximately its freely fed weight.

TRAINING

Rats were trained and tested 5 days per week, Monday through Friday as described previously (Terry et al. 1996, 1997) in operant chambers that were enclosed in ventilated, sound and light attenuated cubicles (dimensions, 27.9 × 29.2 × 30.5 cm; Coulbourn Instruments). Each chamber was fully computer automated with levers centered on each side of a feedbox. Each lever, one on the right and one on the left, was depressed to earn food pellet rewards. The animals were trained to discriminate between a light and a tone stimulus; that is, a reward is provided only on the right side following a trial that begins with the presentation of a light, whereas following a tone, only a response to the left side was rewarded. Immediately following the stimulus, a delay associated with an equal number of light and tone stimuli was presented repetitively to comprise a daily test session of 64 trials. During the delay, the rats were sequestered behind retractable doors that remained closed to prevent access to the levers. At the end of the delay, the doors quickly opened, allowing access for lever selection by the rat. The doors remained open for 5 sec to allow time for the rat to choose a lever and if a correct choice was made, consume its reward. The number of seconds taken to depress a lever was recorded as the "choice latency." Finally, the doors were gently closed for a total intertrial interval of 10 sec. If an incorrect choice was made, no reward was given, and the next trial was initiated.

The delays in the DSDT began at 1 sec and were increased to 3 sec and then to 5 sec when accuracy approached 90%. Once the animals learned to discriminate correctly during trials involving a 5-sec delay (criterion = >75% accuracy), for at least 10 consecutive sessions, i.p. saline injections (1.0 ml/kg) were initiated. Daily saline administration continued until baseline performance was unaffected by injection (typically, 3–5 sessions). Thereafter, saline administration was continued throughout the course of the study on non-drug days. Saline or PF-68 (in PBS) was administered i.p., 30 min prior to DSDT testing.

CONDITIONED TASTE AVERSION

A conditioned taste aversion (CTA) to a novel sweet-tasting solution (two-bottle paradigm) was used as described previously (Prendergast et al. 1997) except that a 0.1% saccharin solution was used in the place of sucrose. At 1000 hr on the day of conditioning (day 1), water bottles were removed from the home cages of all animals. Following 4 hr of water deprivation (at 1400 hr), rats were given access for 30 min to two bottles attached to the front of their home cages. One bottle contained 200 ml of a novel 0.10% (wt/vol) sodium saccharin solution, and the other contained 200 ml of tap water. The position of saccharin and water bottles on the front of cages was counterbalanced on each day of presentation. Immediately following the end of the 30 min, water bottles were removed, and the amount of the different solutions consumed was measured (in ml). Each animal then received an injection (i.p.; $N = 7–8$ for all groups) of saline or PF-68 (200, 800, or 2400 μg/kg). Following injection, water bottles filled with tap water were placed on the front of home cages.

At 1000 hr of the following day (day 2), water bottles were removed from the home cages of all animals. Four hours later, rats were again given access to the two solutions for 30 min. Following this test of CTA, rats received an injection of saline or drug identical to that received the previous day. Water bottles filled with tap water were then returned to all home cages. Water deprivation and testing were completed in an identical manner on the following day (day 3). No injections were given following consumption on day 3.

ROTOROD TEST

Rats were injected with PF-68 or saline and tested after 30 min. Each animal was placed on an accelerating rotorod (Ugo Basile, Italy) that used a rotating treadmill that accelerated from 4 rpm to 40 rpm over a 5-min period. Animals were given one trial on the apparatus on the day prior to the experiment. On the day of the experiment, the total number of seconds maintained on the rotorod was recorded.

LOCOMOTOR ACTIVITY

Rats were injected with PF-68 or saline and tested for changes in locomotor activity after 30 min. Automated open field locomotor activity was measured using an Omnitech Digiscan (model CC-DIGIO) optical animal activity monitoring system that uses horizontal and vertical banks of photo beam sensors to monitor several categories of animal movement with time. Each animal was placed in a clear test cage (40 × 40 × 30 cm), and the trial initiated immediately. Locomotor activity was monitored continuously for 30 min, and the data were accumulated and processed in a spreadsheet format. The following parameters were recorded: horizontal activity, movement time, number of stereotypy movements, and vertical activity. A complete description and definition of each parameter has been published previously (Sanberg et al. 1985).

STATISTICAL ANALYSES

Comparisons between the means of several groups were performed by using one- or two-way ANOVA (with repeated measures when appropriate), and the differences considered significant at the $P < 0.05$ level. Post-hoc comparisons were subsequently made according to the Student–Newman–Keuls method. If data sets failed tests of normality or of equal variances, the nonparametric method of Kruskal-Wallis (analysis of variance on ranks) was used with post-hoc comparisons made according to Dunn's method. In the case of passive avoidance training, learning frequencies (number of animals not crossing vs. those crossing into the unsafe side of the chamber) were also compared using χ^2 analyses (2 × 4 contingency table for comparisons across all groups and post-hoc 2 × 2 contingency table analysis for individual group comparisons). In the case of cell culture experiments, statistical analysis of the experimental data was perfermed using the Student's *t*-test in the case of nicotine stimulated [^{3}H]NE uptake and [^{3}H]NE release experiments, whereas a paired *t*-test was used to analysis FM I-43 dye release.

Results

BRAIN PENETRATION STUDY

^{14}C-Labeled PF-68 administered by peripheral (i.p.) injection (2400-μg/kg) was detectable both in whole brain homogenates as well as homogenates taken from several selected brain regions (i.e., hippocampus, frontal cortex, corpus striatum, cerebellum). Concentrations were quite simi-

lar for whole brain homogenates as well as in individual regions with concentrations ranging from 0.11 to 0.15 nmole/mg SDS extractable protein. Thus, no regional differences in PF-68 penetration into the brain were detected.

IN VITRO EFFECTS OF PF-68 ON NEUROTRANSMITTER UPTAKE AND RELEASE

In cultured PC-12 cells, exposure to a solution of 1% (wt/vol) PF-68 for a period of 5 min that resulted in a dose of 0.3 nmoles PF-68/mg SDS extractable protein, significantly ($P < 0.005$) reduced [^{3}H]NE uptake (Fig.1A) from the tissue culture medium indicating that endocytosis of the neurotransmitter substance was inhibited by the presence of PF-68 in the membrane. In cultured PC-12 cells that had been loaded previously with [^{3}H]NE using KCl depolarization prior to PF-68 exposure (i.e., selective loading of the depolarization-sensitive intracellular vesicle population—"synpatic vesicles"), PF-68 significantly ($P < 0.03$) reduced the amount of [^{3}H]NE released as a consequence of nicotine exposure (Fig. 1B). Furthermore, if the aqueous-phase fluorescent dye FM I-43 was loaded into the depolarization sensitive intracellular vesicle population of PC-12 cells, rather than [^{3}H]NE, a similar significant ($P < 0.005$) reduction in the amount of FM I-43 released upon nicotine exposure was again detected (Fig. 1C). As nicotine would be expected only to cause release of [^{3}H]NE or FM I-43 contained in synaptic vesicles mobilized to fuse with plasma membrane upon activation of the nAChR, these data suggest that the presence of PF-68 in the plasma membrane affects the ability of the nAChR to bring about neurotransmitter release upon nicotine stimulation.

PASSIVE AVOIDANCE TESTING

Median step-through latencies to enter the dark chamber and learning frequencies during retention trials after the various doses of PF-68 are illustrated in Figure 2. Highly significant treatment effects were apparent for both the latency data (Kruskal-Wallis test, $H = 19.23$, $P < 0.001$) and frequency data (χ^2 analysis, $\chi^2 = 16.87$, $P < 0.001$). Post-hoc analyses revealed that when compared with saline associated performance, PF-68 reduced median latencies to cross (and reduced the frequency of animals that learned not to cross) into the unsafe portion of the chamber ($P < 0.05$) in a dose-dependent manner.

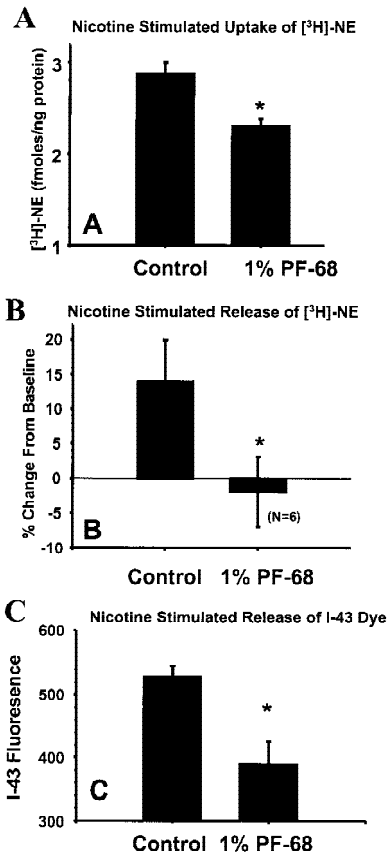

Figure 1: (*A*)[^{3}H]NE uptake into differentiated PC-12 cells. [^{3}H]NE uptake (radioactivity) was measured in Triton X-100 solubilized PC-12 cells by liquid scintillation counting in the absence (control) or presence of 1% PF-68 ($N = 24$ individual cultures per condition). (*B*) Nicotine induced [^{3}H]NE release in PC-12 cells. Release of [^{3}H]NE induced by 200 μM nicotine was measured in PC-12 cells in the absence (control) or presence of 1% PF-68 ($N = 6$ individual cultures per condition). (*C*) FM I-43 release assay. Nicotine stimulated FM I-43 dye release from PC-12 cells was measured before (control) and after exposure to 1% PF-68 in the same cultures ($N = 12$ individual cultures per condition). (*) Significantly different ($P < 0.05$) from baseline levels.

WATER MAZE TESTING

HIDDEN PLATFORM TEST

The latencies and swim distances required to locate a hidden platform in the water maze for days 1–4 are illustrated in Figure 3, A and B. Statistical comparisons of latencies across the four groups revealed the following results: group effect [$F(3,47) = 17.8$, $P < 0.0001$]; day effect [$F(3,9) = 48.2$, $P < 0.0001$]; group × day interaction [$F(141,203) = 5.28$, $P < 0.0001$]. Similar re-

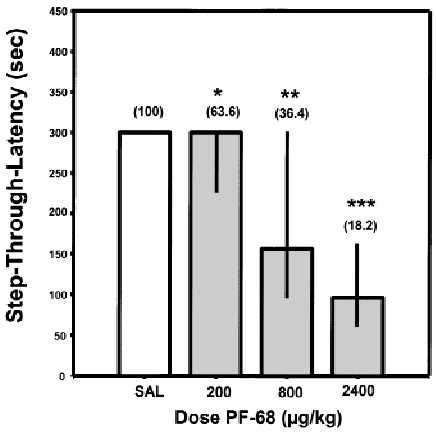

Figure 2: Passive (inhibitory avoidance) effects of saline, or PF-68 at 200, 800, and 2400 µg/kg on 48-hr passive (inhibitory) avoidance training. Bars represent median step-through latencies (in sec) to enter the dark chamber and the range between the 25th and the 75th percentiles (vertical lines). The numbers in parentheses represent learning frequencies (% of rats having learned not to cross into the unsafe portion of the chamber). The actual ratios of animals learning not to cross were as follows: saline, 11 out of 11; PF-68 200, 7 out of 11; PF-68 800, 4 out of 11; and PF-68 2400, 2 out of 11. (*) Significantly different ($P < 0.05$) from saline performance; (**) significantly different from saline and PF-68 200 mg/kg associated performance; (***) significantly different from saline, PF-68 200, and PF-68 800 µg/kg associated performance. $N = 12$ rats per group.

sults were evident when swim distances were analyzed. Under saline conditions the rats learned to locate the hidden platform with progressively shorter latencies across the 4 days of training. Post-hoc comparisons indicated that the 2400 µg/kg dose of PF-68 significantly increased mean latencies (designated by * in Fig. 3A,B, $P < 0.05$) on days 2–4 and the lower doses (200 and 800 µg/kg) also increased latencies and/or swim distances on specific days.

TRANSFER TEST (PROBE TRIALS)

Drug effects on spatial bias indicated by the percent of time spent and distance swum in the quadrant in which the platform had been located during the first 4 days of testing are illustrated in Figure 4, A and B. Significant drug effects were observed [$F(3,44) = 3.56$, $P = 0.022$], and post-hoc analyses revealed that the 2400-µg/kg dose of PF-68 reduced the bias for the correct quadrant significantly below saline performance levels. The 800-µg/kg dose of PF-68 also decreased performance; however, the difference did not reach the required level of significance ($P > 0.05$).

We also analyzed all the groups individually in the transfer test (both as percent total distance and time swum in the target quadrant) by one-sided, (two-tailed) *t*-tests and all of the groups performed significantly ($P < 0.05$) above the chance level of 25%. It is somewhat perplexing as to why the rats given the highest dose of PF-68 performed at this level when they showed little evidence of learning during the standard (first 4 days) water maze testing. It is important to note that although the rats in the high dose group showed little evidence of learning to locate the 10-cm platform in the first 4 days of testing, they did spend more time in the target quadrant as the experiments progressed toward day 4. Thus, some level of learning was apparent because a bias toward the quadrant that held the platform was present even though it did not result in successful location of the platform during the 90-sec test period.

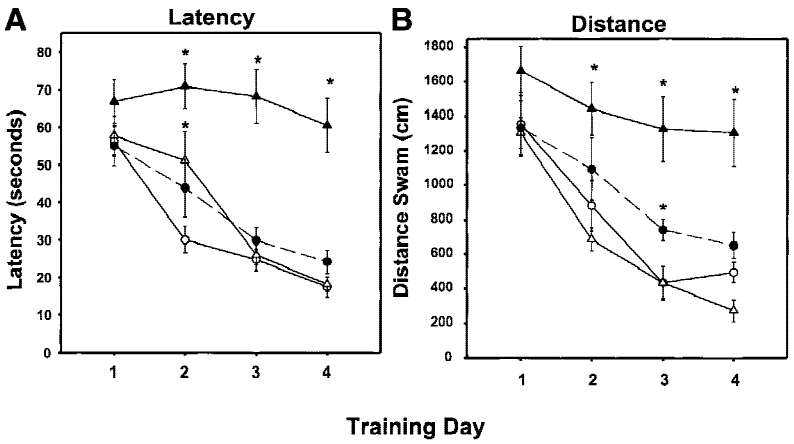

Figure 3: Spatial navigation learning in the MWM. (*A*) Effects of PF-68 (at three doses) on latencies to find a hidden platform in the water maze on 4 consecutive days of testing compared with saline. (*B*) Effects of PF-68 on distance swum (in cm) to find a hidden platform in the water maze on 4 consecutive days of testing compared with saline. Each point represents the mean ± S.E.M. of four trials per day. (*) Significantly different from saline control value ($P < 0.05$). $N = 12$ rats per group. (○) Saline; (△) 200 µg/kg PF-68; (●) 800 µg/kg PF-68; (▲) 2400 µg/kg PF-68.

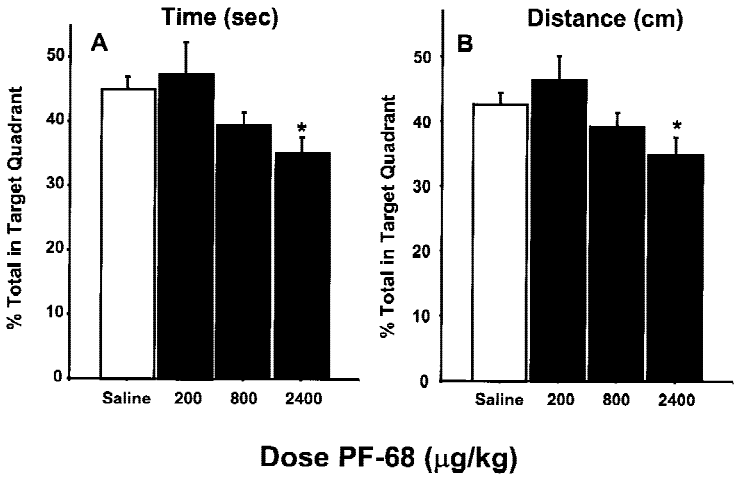

Figure 4: Performance of the water maze probe trials in rats administered saline or PF-68 (three doses). Each bar represents the percentage (±S.E.M.) of the total time spent (*A*) or distance swum (*B*) in the quadrant where the platform was located in the previous 4 days of testing.

MOTOR EFFECTS

Motor effects of PF-68 as illustrated by the time maintained on an accelerating rotorod and by swim speeds in the water maze (day 1 and day 5) are presented in Figure 5. Locomotor activity was also assessed in an automated open field activity monitoring system (Table 1). In each case, PF-68 had no significant effects in the particular task.

VISUAL ACUITY TEST

A general estimate of visual acuity was assessed by allowing the rats to locate a highly reflective (visible) platform after standard water maze testing was completed. The mean time required to locate the platform under saline conditions was 28.0 ± 5.7 sec. None of the three doses of PF-68 affected the mean time to locate the platform [$F(3,46) = 0.421$, $P = 0.739$] (data not shown).

DSDT

The effects of PF-68 on DSDT accuracy are represented in Figure 6 [dose effect, $F(5,55) = 0.386$, $P = 0.857$]. No statistically significant differences between saline and PF-68 performances were observed. There were also no significant effects of the various doses of PF-68 on DSDT choice latencies (i.e., the latency in seconds taken to select a lever). Latencies typically ranged from 0.8 sec to 1.3 sec (data not shown).

CONDITIONED TASTE AVERSION

A comparison of control animal's water and saccharin consumption was made on each day of testing to confirm a preference for saccharin, indicating the salience of the novel sweet taste. Animals that failed to consume 3 or more ml of saccharin solution on the conditioning day were excluded from further testing to assure that all animals tested subsequently had significant exposure to the novel taste. On the conditioning day, all animals displayed a significant preference for the saccharin solution, as compared with water [$F(1,27) = 5.71$, $P < 0.05$]. Similarly, saccharin consumption was greater than water consumption on both days 2 (7.50 ± 0.93 vs. 5.38 ± 0.75 ml, respectively) and 3 (8.25 ± 1.68 vs. 4.63 ± 0.71 ml, respectively) of aversion testing. Saccharin consumption in PF-68 treated animals on both days 2 and 3 was similar to that of controls (Fig. 7). No avoidance of the novel saccharin taste was observed on either day, indicating the lack of a malaise-induced

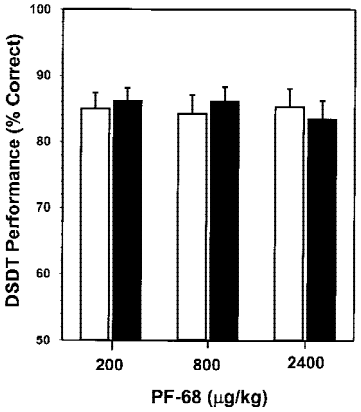

Figure 5: Performance of the DSDT. DSDT performance in 12 rats, 30 min following the i.p. administration of three doses of PF-68 (solid bar) or saline (open bar). Each point represents the mean (% correct) over 64 trials per session ± S.E.M.

Table 1: *Effects of PF-68 on open field locomotor activity in rats*

Activity	Saline	PF-68 (200)	PF-68 (800)	PF-68 (2400)
Horizontal activity	13,368 ± 1214	13,500 ± 1115	13,427 ± 778	14,258 + 762
Movement times	731 ± 68	765 ± 52	741 ± 49	804 + 41
Number if sterotypy	269 ± 12	279 ± 11	268 ± 12	267 + 10
Vertical activity	3308 ± 192	3538 ± 198	3520 ± 234	3421 + 164

Values above are means ± S.E.M. for 10 rats per group. The numbers in parentheses are i.p. doses in μg/kg.

avoidance of the saccharin solution. Water consumption remained stable over the 3 days of testing (4–5 ml each day for each group; Fig. 7, inset). As a compensatory increase in water consumption is observed in water-deprived animals during the development of a taste aversion, these data also suggest the absence of a learned aversion to the saccharin solution.

Discussion

Peripheral administration of the membrane ordering agent PF-68 in rats resulted in significant levels of compound in brain tissue. In cell culture experiments, direct exposure of NGF-differentiated PC-12 cells to PF-68, at concentrations approximating that achieved in brain tissue after systemic administration (0.15 nmoles/mg SDS extractable protein in vivo vs. 0.3 nmoles/mg SDS extractable protein in vitro), resulted in the inhibition of both the uptake of NE and the release of the neurotransmitter when the cells were exposed to nicotine. Furthermore, the peripheral administration of PF-68 resulted in a reduction in learning and memory consolidation [passive avoidance and Morris water maze (MWM) studies] in rats that were naive to memory testing but did not appear to affect retrieval of sensory cues in a well-learned working memory task (DSDT). We selected the passive avoidance procedure as initial screening paradigm for potential mnemonic effects of PF-68 because it is simple to perform and easy to accomplish in a short period of time. Further testing was performed in a spatial learning task, the MWM, that utilizes various mnemonic processes including sophisticated information acquisition, processing, consolidation, retention, and retrieval (for review, see McNamara and Skelton 1993). Place learning in rodents is highly dependent on hippocampal function and other medial temporal lobe structures known to be of importance in human learning and memory processes (McDonald and White 1995). We also evaluated PF-68 in the DSDT paradigm to assess effects on working memory and to better simulate memory tasks used in humans. Although there are procedural differences between rodent discrimination tasks such as DSDT and primate (human and nonhuman) delayed matching tasks, they do share several important features including conditional (modality) discriminations with delay

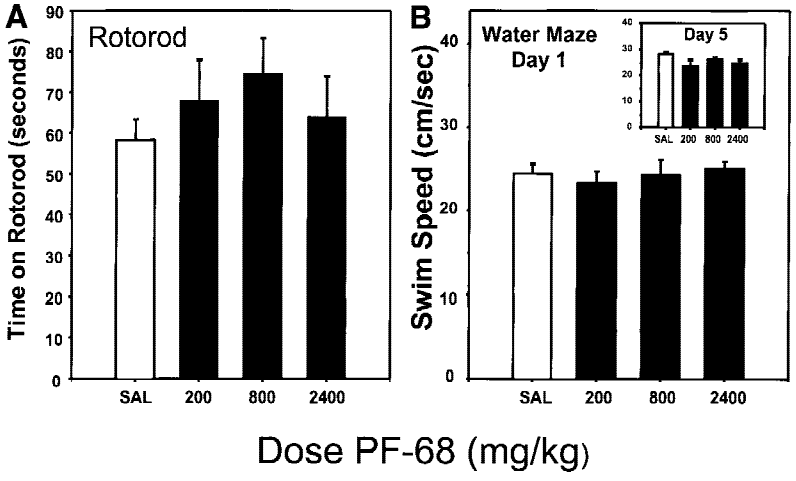

Figure 6: Motor effects of PF-68. (*A*) Adult Wistar rats were tested on the accelerating rotorod test 30 min after i.p. administration of saline or PF-68. Data presented are mean time maintained on the rotorod ± S.E.M. (*B*) Swim speeds (cm traveled/sec ± S.E.M.) in the water maze test were on days 1 and 5. $N = 10–12$ rats per group.

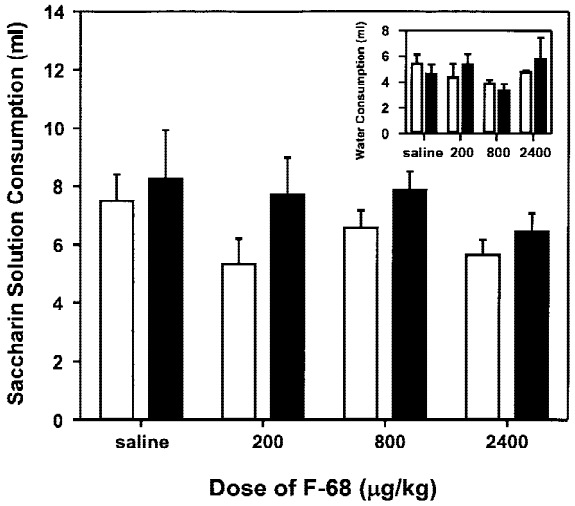

Figure 7: Conditioned taste aversion. Saccharin solution (0.10%) and water consumption (*inset*) of animals treated with F-68 (200, 800, or 2400 μg/kg) on days 2 (open bar) and 3 (solid bar) after pairing of drug administration with the novel saccharin solution. Data represented as mean milliliter consumed ±S.E.M. No significant differences in consumption between drug- and saline-treated animals were observed. $N = 7–8$ rats per group.

intervals and several aspects of working and reference memory including attention, information processing, storage and retrieval, etc. (Squire et al. 1988; Bushnell 1990; Dunnett and Martel 1990). Furthermore, a significant body of evidence suggests that several working memory processes assessed in delayed response tasks designed for rodents as well as primates use analogous regions of the medial prefrontal cortex (for review, see Granon et al. 1995).

No PF-68 related changes were observed in swim speeds or visual acuity in water maze experiments, performance of an accelerating rotorod procedure, or in tests of general (open field) locomotor activity. Moreover, PF-68 did not initiate a CTA after peripheral administration. CTA is a learned taste avoidance developed after associative pairing of a novel taste cue and nausea or a more general gastrointestinal malaise and is used frequently to assess the ability of pharmacologic agents to induce such states (Garcia and Koelling 1966). Collectively, these results suggest that PF-68 induced deficits in memory without confounding motor, sensory, or gastrointestinal effects at the tested doses. Furthermore, these data suggest that direct manipulation of neuronal PMO by a compound that does not directly interact with membrane proteins associated with transmembrane signaling but, rather, modifies the biophysical properties of the three dimensional lipid matrix in which these protein reside (Ramirez and Mutharasan 1990; Clarke et al. 1995), can have profound effects upon neuronal function at not only the cellular level but at the integrated systems level required for complex memory formation.

Our experimental observations regarding the effect of PF-68 on neurotransmitter uptake and release in cultured cells suggest that one possible explanation of the dose-dependent, detrimental effects of this membrane ordering agent upon cognitive function in the whole animal may lie in the disruption of similar events in vivo. One experimental observation that we have made is that an acute effect of increasing neuronal PMO is the disruption of the neuronal communication required for the formation of new structural memory (i.e., neurotransmitter release occurring at synapses that are in the process of being recruited into new memory pathways), rather than disruption of signaling in well-established pathways. This contention is supported by the time course of the effects seen in the whole animal, namely that a 30-min exposure to PF-68 results in a dose-dependent decrement in memory formation but does not effect memory retrieval in a well-learned task. It is well accepted that multiple repetitions of a new task result in the formation of neural memory networks that have a structural basis in the brain (i.e., a physical localization within the brain as a result of structural connections between the constituent neurons making up that network). These structural neural networks responsible for memory are formed only after multiple, ordered firings of the neurons within the pathway. If, however, a decrease in PMO after PF-68 administration results in a disruption of synaptic transmission, either by inhibition of neurotransmitter release or by inhibition of neurotransmitter reuptake as we have demonstrated in vitro (Fig. 1), the formation of new "structural memory" pathways by functionally refractive neurons would be less efficient. Conversely, in a well developed memory pathway consisting of numerous structural interactions between its constituent neurons, a reduction in synaptic transmission would have much less of an effect on the function of the network as whole than on individual neurons not yet integrated into a network.

Evidence to support the hypothesis that an increase in PMO (i.e., a decrease in plasma mem-

brane fluidity) can effect transmembrane signaling comes from several different areas. First, increased amounts of cholesterol in the plasma membrane of a variety of different cell types, both in vitro and in vivo, result in a concomitant increase in PMO and alteration in the fidelity of numerous transmembrane signaling events, including those associated with neuronal transmission (for review, see Bastiaanse et al. 1997). Second, increased cholesterol content and the subsequent increase in PMO of skeletal muscle sarcolemma after feeding rats a high fat diet results in a reduction in the sensitivity of this tissue to circulating anabolic factors, such as insulin and insulin-like growth factor I (Igf1) (Liu et al. 1994, 1995; Storlien et al. 1996). Decreased growth factor sensitivity in these animals has been associated with the inability of the growth factor receptor to undergo the appropriate conformational changes required for efficient transmembrane signaling of the growth stimulus. This effect has been referred to as molecular freezing of the receptor protein in the three-dimensional lipid matrix of the sarcolemma. This phenomenon can be caused by a number of alterations in the lipid matrix of the plasma membrane, including increased cholesterol content, increased saturated fatty acid content, and increased cross-linking of the lipid membrane matrix due to free radical-induced lipid peroxidation, all of which result in an increase in PMO (Liu et al. 1994; Choi and Yu 1995; Storlien et al. 1996). More importantly, the decrease in skeletal muscle sensitivity to insulin and IGF-1 observed after an increase in PMO due to cholesterol enrichment of the sarcolemma could be reversed if the animals were fed a diet high in omega-3 fatty acids. The omega-3 fatty acids were shown to substitute into the sarcolemma and bring about a decrease in PMO (i.e., an increase in plasma membrane fluidity) that was paralleled by a return to normal levels of insulin and Igf1 sensitivity in the muscle (Liu et al. 1994, 1995). Third, increased PMO brought about by either cholesterol enrichment of the plasma membrane or exposure to a membrane ordering agent results in an increase in mechanical shear-induced plasma membrane wounding (Clarke and McNeil 1992; Clarke et al. 1995). It is interesting to note that the cellular processes required for membrane resealing of a plasma membrane wound (i.e., a calcium-dependent mobilization of an intracellular membrane pool that fuses with the plasma membrane at the site of the membrane wound) (Miyake and McNeil 1995; Terasaki et al. 1997) have been shown to be similar to those associated with synaptic vesicle fusion during neuronal transmission (Steinhardt et al. 1994). These data suggest that conditions that inhibit membrane wound resealing and hence increase susceptibility to mechanically induced plasma membrane wounding (i.e., increased PMO) may also inhibit synaptic transmission.

PF-68, like cholesterol, is also an amphipathic molecule capable of intercalating into the plasma membrane (Clarke and McNeil 1992; Clarke et al. 1995) and brings about a decrease in PMO in a similar fashion to that observed after cholesterol enrichment of the plasma membrane (Clarke et al. 1995). It is interesting to speculate that alterations in neuronal membrane cholesterol content, with the concomitant increase in PMO, may have similar effects on neuronal transmission to that seen with acute PF-68 administration. Experimental evidence to support this contention comes from studies using a striatal slice superfusion system in which muscarinic AChR sensitivity (Joesph et al. 1993) and K^+-evoked release of dopamine (Joesph et al. 1995) was reduced in tissue from young rodents if the sections were incubated in cholesterol. Conversely, in tissue from aged rodents, muscarinic AChR sensitivity and K^+-evoked release of dopamine were both increased if the tissue was incubated with membrane disordering agent, *S*-adenyl-L-methionine (Joesph et al. 1993, 1995).

The possibility that alterations in neuronal membrane PMO per se could bring about changes in cognitive function is intriguing. For example, the basis of antioxidant therapy in the protection of cognitive function may lie in preventing the generation of reactive groups on the lipid constituents within the membrane matrix, thereby preventing an increase in neuronal PMO by reducing the number of cross-links between membrane constituents (Choi and Yu 1995; Stoll et al. 1996). The finding that neuronal membranes from aged animals exhibit a higher degree of PMO (i.e., decreased plasma membrane fluidity) than neuronal membranes from younger animals (Joesph et al. 1995; Stoll et al. 1996) suggests that age-related alterations in neuronal function may have their basis in alterations in the biophysical properties of neuronal membranes that then effect the function of proteins, such as AChR, embedded in those membranes.

In conclusion, the results of this study indicate that the plasma membrane ordering agent, PF-68, interferes with neurotransmitter uptake and release in vitro and induces deficits in the perfor-

mance of certain memory tasks without peripheral motor, sensory, or gastrointestinal side effects. The data indicate that an increase in neuronal PMO may have profound effects on neurotransmitter function as well as learning and memory processes. Furthermore, compounds such as PF-68 may also offer novel tools for studying the role of neuronal PMO in mnemonic processes and changes in PMO resulting from age or AD.

Acknowledgments

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